Anti-EFNA4 Calicheamicin Conjugates Effectively Target Triple-Negative Breast and Ovarian Tumor-Initiating Cells to Result in Sustained Tumor Regressions

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Abstract

**Purpose:** Triple-negative breast cancer (TNBC) and ovarian cancer each comprise heterogeneous tumors, for which current therapies have little clinical benefit. Novel therapies that target and eradicate tumor-initiating cells (TIC) are needed to significantly improve survival.

**Experimental Design:** A panel of well-annotated patient-derived xenografts (PDX) was established, and surface markers that enriched for TIC in specific tumor subtypes were empirically determined. The TICs were queried for overexpressed antigens, one of which was selected to be the target of an antibody–drug conjugate (ADC). The efficacy of the ADC was evaluated in 15 PDX models to generate hypotheses for patient stratification.

**Results:** We herein identified E-cadherin (CD324) as a surface antigen able to reproducibly enrich for TIC in well-annotated, low-passage TNBC and ovarian cancer PDXs. Gene expression analysis of TIC led to the identification of Ephrin-A4 (EFNA4) as a prospective therapeutic target. An ADC comprising a humanized anti-EFNA4 monoclonal antibody conjugated to the DNA-damaging agent calicheamicin achieved sustained tumor regressions in both TNBC and ovarian cancer PDX in vivo. Non-claudin low TNBC tumors exhibited higher expression and more robust responses than other breast cancer subtypes, suggesting a specific translational application for tumor subclassification.

**Conclusions:** These findings demonstrate the potential of PF-06647263 (anti–EFNA4-ADC) as a first-in-class compound designed to eradicate TIC. The use of well-annotated PDX for drug discovery enabled the identification of a novel TIC target, pharmacologic evaluation of the compound, and translational studies to inform clinical development. *Clin Cancer Res;* 21(18): 4165–73. ©2015 AACR.

Introduction

One in eight women in the United States will develop breast cancer—17% of whom will be diagnosed with triple-negative breast cancer (TNBC) because their tumors lack HER2, estrogen receptor (ER), and progesterone receptor (PR; ref. 1). TNBC has defied attempts at targeted therapy, in part because of the absence of obvious, tractable targets. TNBC has also exhibited inconsistent response and inadequate responses to chemotherapy and antiangiogenic agents. As a consequence, there are currently no treatment guidelines for TNBC (2, 3). In ovarian cancer, platinum-based therapy has provided modest benefit, and the recent approval of bevacizumab marked the first improvement in treatment in 15 years (4–9).

Amaising evidence supports the hypothesis that tumor growth, resistance to therapy, and disease recurrence are driven by a subpopulation of tumor cells known as tumor-initiating cells (TIC). Novel therapies that target and eradicate TIC could significantly improve survival endpoints for patients. The precise identification and characterization of TIC have proven difficult, particularly because conventional cancer cell lines do not faithfully recapitulate intratumoral cellular heterogeneity (10, 11). Solid tumor cell subpopulations significantly enriched for TIC were first identified in breast cancer; however, the CD44+/CD24− phenotype used in that study did not enrich TIC in all patient-derived xenografts (PDX) tumors, as CD24− cells were tumorigenic in the T7 PDX (12). Indeed, tumor types defined by tissue of origin encompass a spectrum of histologic and molecular subtypes, and one TIC phenotype is not likely to be broadly applicable. To date, TIC identities have not been specifically ascribed to...
Translational Relevance

Triple-negative breast cancer (TNBC) has been recalcitrant to targeted therapies and exhibited inconsistent and inadequate responses to chemotherapy and antiangiogenic agents. We have identified a novel tumor antigen, EFNA4, which is broadly overexpressed in TNBC, as well as ovarian cancer. We developed an anti-EFNA4 antibody–drug conjugate (ADC) that achieved sustained tumor regressions in TNBC patient-derived xenografts (PDX) and reduced the frequency of tumor-initiating cells, suggesting that the ADC may induce durable responses in the clinic. Translational studies executed using a panel of well-annotated PDX tumor models provided additional insight into which subsets of patients are more likely to respond to the therapy in the clinic. This anti–EFNA4-ADC is currently in clinical trials informed by the preclinical and translational research presented here.

disease subtypes, which may explain the discrepant results both across and within research groups attempting to apply one TIC phenotype to all subtypes. PDXs have emerged as powerful preclinical models because, in contrast to cell lines, they maintain the karyotype, cellular heterogeneity, and architecture of parental tumors (13, 14). Utilization of well-annotated PDX tumor libraries should also enable the identification of TIC in specific tumor types, the discovery of therapeutic targets, and the in vivo evaluation of novel compounds while remaining cognizant of the translational relevance across different tumor subtypes (11, 15).

Ephrin receptors (Eph) comprise the largest family of receptor tyrosine kinases in the human genome and modulate signaling pathways that impact cell fate decisions during embryogenesis and adult tissue homeostasis (16). Reverse signaling to the ligand (Ephrin)-expressing cell can accompany forward signaling to the Eph-expressing cell; however, the mechanisms underlying these signaling cascades remain unclear (17–19). The Ephrin/Eph family is implicated in breast cancer, where expression of several members inversely correlates with survival (18, 20). These observations, coupled with the established role of Eph signaling in cancer, have resulted in numerous targeted therapeutics that have advanced to clinical trials: the majority of which are tyrosine kinase inhibitors (TKI; refs. 17, 19, 21–23). Vast functional pathways that impact cell fate decisions during embryogenesis.

Materials and Methods

PDXs

Following Institutional Review Board approval at the Cooperative Human Tissue Network (CHTN) and National Disease Research Interchange (NDRI), patients with breast and ovarian cancer consented to enable live tumor specimen collection. Each tumor specimen was coated with Matrigel (BD Biosciences) and implanted into 6- to 10-week-old NOD-scid mice (obtained from Harlan or Charles River Labs) near the mammary fat pad. Research animals were housed and handled according to Institutional Animal Care and Use Committee (IACUC)-approved protocols and procedures in accordance with American Association for Laboratory Animal Science recommendations. Each PDX was authenticated as unique and matching the primary tumor specimen with the Ion AmpliSeq Sample ID Panel (4779970; Life Technologies; Supplementary Table S1).

Resulting freshly resected xenograft tumors were dissociated to a single-cell suspension as described previously (12, 33). At each passage of tumor propagation, human epithelial origin was confirmed by positive flow cytometry staining using anti-human ESA (Clone 9C4), and negative staining with anti-mouse CD45 (Clone 30-F11) and anti-mouse H-2Kd (Clone SF1-1.1) antibodies (all from BioLegend). Tumor measurements were recorded one or two times per week using digital calipers, and tumor volume was estimated using the equation $V = \frac{(A \times B^2)}{2}$, where $A$ is the long axis and $B$ is the short axis.

In vivo efficacy studies

CoHORTS of tumor-bearing mice (140–180 mm$^3$) were randomized into study groups of 6 to 10 based on the number of available mice. The IDBS electronic notebook statistical package, Biobook, was used for automated animal randomization. Animals were dosed by intraperitoneal injection (or intravenously for 144580) twice a week for 4 cycles with ADC, or once a week for 2 cycles with 1.5 mg/kg doxorubicin for breast PDX tumors or 5 mg/kg Cisplatin for ovarian PDX. Study groups were followed until either individual mice or entire cohort measurements reached 1,200 mm$^3$, at which point sacrifice was indicated in accordance with approved IACUC protocols. Tumor regression was defined as a reduction in mean tumor volume after dosing. In cases where tumors regressed, time to progression (TTP) was determined to be the number of days between the first dose and the time at which mean tumor volume significantly increased (regrew) after regression.

TIC frequency assay

PDX tumor–bearing mice were treated with PF-06647263 or control ADC, and tumors were harvested at day 21 (BR13) or day 12 (BR22) after the first dose, based on when tumors were starting to regress. Tumors were harvested, dissociated, and stained as described above. Three tumors per treatment group were pooled, and live human tumor cells (murine Lineage$^-$ ESA$^+$) were isolated by FACS, counted, and implanted into naive animals in
Results

TNBC TICs are significantly enriched among CD324⁺ cells

To expedite the discovery and development of breast cancer therapeutics while remaining cognizant of disease subtype, we established a PDX tumor bank from 13 TNBC patients, 10 were the non-Claudin low molecular subtype and 3 were Claudin-low (34, 35). Upon careful phenotypic profiling of CD24, a marker thought to demarcate TIC in breast cancer (12), it became apparent that TNBC PDX tumor cells uniformly expressed the antigen (Fig. 1A and B), and thus it would not add utility for TIC enrichment. In contrast, phenotypic profiling of dissociated tumor cells identified E-cadherin (CD324) as heterogeneously expressed (Fig. 1B). Isolation and transplantation of cells based on their differential expression of CD324 demonstrated that only CD324⁺ cells, but not their CD324⁻ counterparts, were able to efficiently perpetuate tumors that replicated the phenotypic heterogeneity of their parental tumors (Fig. 1C and D) and could be serially transplanted. Importantly, fully heterogeneous tumors were initiated with as few as 50 implanted cells, whereas TIC frequency within the CD324⁻ subpopulation was within error of the false positive expectation resulting from a 0.5% cell impurity profile of isolated cells (Supplementary Table S2). The above agnostic and empirical approach to determine TIC markers based on in vivo tumorigenicity revealed that the conventional TIC phenotype CD44⁺ CD24⁻ was not applicable in this subtype of breast cancer, since all tumor cells were CD24⁺, and that CD324 effectively enriched TIC in TNBC.

EFNA4 is elevated in non-Claudin low TNBC TIC

Leveraging the above enrichment of TNBC TIC, whole transcriptome sequencing was performed using isolated CD324⁺ TIC and CD324⁻ nontumorigenic (NTG) cells from seven breast PDX tumor models of various subtypes, including Claudin low (CL) and non-Claudin low (non-CL) TNBC. Notably, EFNA4 expression was identified as elevated in TIC versus both NTG cells and normal tissues (Figs. 2A and B), and further validated by qRT-PCR in those samples submitted for whole transcriptome sequencing, and separately in TIC and NTG cell populations isolated from additional PDX tumors (data not shown). Since EFNA4 expression was clearly observed upon focusing on the TNBC subtype of breast cancer and upon enriching for TIC, and has not been previously noted as a target of interest in breast cancer, we asked whether elevated expression was detectable in the context of bulk breast PDX tumors and/or among clinical patient tumor specimens. EFNA4 mRNA expression was elevated versus normal breast tissue and 11 other normal tissues and was generally higher in the non-Claudin low subtype of TNBC (n = 10) versus other breast cancer subtypes, including Claudin low TNBC (Fig. 2C). Upon applying the PAM50 gene signature to The Cancer Genome Atlas (TCGA) data to better characterize the dataset according to probable subtypes (36), elevated expression of EFNA4 in TNBC tumors was confirmed compared with normal adjacent breast and breast tumors of non-TNBC subtypes (Fig. 2D). A correlation between EFNA4 mRNA and DNA copy number was also observed (n = 110; r = 0.379; P < 0.0001; Fig. 2E), suggesting a genetic basis for EFNA4 overexpression in at least some cases. Surprisingly, 25.5% of breast tumor specimens had notable EFNA4 copy-number gain (n ≥ 2.5), with a slightly higher incidence in TNBC (Supplementary Fig. S1A; ref. 36). A similar trend was also observed in the METABRIC breast cancer dataset (37), where EFNA4 copy-number gains were observed in 14.3% of samples.

Finally, ELISAs were performed to determine whether elevated EFNA4 mRNA translates into increased protein expression. Analysis of protein lysates from 17 normal organs, 49 primary breast tumor specimens, and 9 TNBC PDX tumor models revealed that EFNA4 protein was elevated not only in TNBC versus normal tissues and other subtypes of breast cancer, but also in the molecularly defined non-Claudin low versus Claudin low subset
EFNA4-targeted antibodies mediate efficient payload delivery

Anti-EFNA4 mAbs were generated and characterized for antigen binding and ability to internalize to deliver payload. First, mAbs were screened for their ability to bind EFNA4, but not related family members EFNA1, EFNA3, or EFNA5 (Fig. 3A). The murine mAb clone mE22 demonstrated specificity for antigen and bound EFNA4 without blocking its interaction with a known receptor, EphA2, in contrast with some other mAbs in the panel (data not shown). mE22 was humanized by grafting the Complementarity-Determining Regions onto a human IgG1 framework, resulting in hE22, which maintained its affinity for human EFNA4 (Supplementary Fig. S2A). Humanized hE22 recognized 293T-EFNA4 cells but not parental 293T cells (Supplementary Fig. S2B and S2C) and fully reacted with cynomolgus monkey EFNA4, but not with murine antigen. Both mE22 and hE22 mediated delivery of the Saporin toxin in piggy-back internalization and cytotoxicity assays, with an EC_{50} of 10 ng/mL (Fig. 3B). The ADC PF-06647263 (anti-EFNA4-ADC) was generated via conjugation of hE22 lysine residues to the AcButDMH-N-Ac-calicheamicin-gamma1 linker-payload with an average drug-to-antibody ratio (DAR) of 4.6 (Fig. 3C; Supplementary Fig. S2D). Surface plasmon resonance (Supplementary Fig. S2E) and cell binding studies (not shown) demonstrated comparable antigen binding by the ADC and unconjugated mAb (Supplementary Fig. S2F).

To evaluate the potency of PF-06647263 and confirm its mechanism of action, cells were treated in vitro and subsequent cell viability was assessed. PF-06647263 elicited antigen- and concentration-dependent cytotoxicity, as exposure to PF-06647263 for 96 hours resulted in cell death (EC_{50} ~ 1 ng/mL), whereas the control ADC comprising a nonbinding mAb with the same linker-payload and DAR did not (Fig. 3D). Furthermore, PF-06647263 did not elicit cytotoxicity against parental HEK293T cells lacking EFNA4 expression (data not shown). Induction of apoptosis consistent with calicheamicin’s mechanism of action was confirmed by the presence of γ-H2AX foci indicative of double-stranded DNA breaks in cells exposed to PF-06647263 for 4 hours (Fig. 3E; ref. 38). In contrast, no foci were observed after treatment with control ADC (Fig. 3E) or unconjugated hE22 (data not shown). Staining with Annexin-V demonstrated the induction of apoptosis by PF-06647263, but not by control ADC (Fig. 3E) or unconjugated hE22 (data not shown). Analogous results were obtained in a pharmacodynamic study in vivo, where mice bearing TNBC PDX tumors were administered one dose of 1 mg/kg PF-06647263 and euthanized 24, 48, and 96 hours later for immunohistochemical analysis. Tumors exhibited PF-06647263 staining at the plasma membrane 24 hours after dosing, and subsequent reduction of PF-06647263 staining was complemented by increased nuclear γ-H2AX foci (Fig. 3F; Supplementary Fig. S3).

PF-06647263 induces significant tumor regression in TNBC xenografts

To evaluate the in vivo efficacy of PF-06647263 against breast PDX tumors versus standard-of-care, naive mice were implanted with 50,000 PDX tumor cells, randomized to 6 to 10 mice per group once tumors reached 140 to 180 mm^3 and treated with PBS, 1.5 mg/kg doxorubicin once a week for 2 weeks, or PF-06647263 or control ADC at various dose levels twice weekly for 2 weeks. Whereas breast PDX tumors were largely nonresponsive to doxorubicin, 0.27 mg/kg PF-06647263 resulted in significant tumor regressions often lasting more than 120 days (Fig. 4A; Table 1). Significant tumor regression and/or tumor growth inhibition of TNBC (Fig. 2F). The above results confirm that, even at the bulk tumor level, elevated EFNA4 gene expression translates to meaningful and detectable increases in EFNA4 protein.
(TGI) was also observed at progressively lower doses, down to 0.036 mg/kg in some models (Table 1). The corresponding exposure levels in humans should be achievable based on the highest nonseverely toxic dose achieved in cynomolgus monkeys in exploratory toxicology studies described below.

In total, 9 distinct BR PDX tumor models were evaluated for their response to PF-06647263. Consistent with EFNA4 expression, the most robust responses were observed in the non-Claudin low TNBC tumors—with effective cures observed in several cases. The expression level of EFNA4 in these PDX models was comparable with that in primary TNBC tumors (Fig. 2F). Notably, the ADC did not elicit responses in tumors that did not express the antigen, and the control ADC did not elicit an antitumor activity in any study.

To confirm that tumor regressions were underpinned by a direct impact on TIC, the tumorigenic potential of cells remaining after PF-06647263 exposure was assessed in an in vivo limiting dilution reimplantation assay. Specifically, several BR13 and BR22 PDX tumor-bearing mice treated with 0.09 mg/kg or 0.1 mg/kg of the control ADC were euthanized 21 or 12 days, respectively, after treatment, following which human tumor cells were isolated and serially transplanted in limiting dilutions. Using Poisson distribution statistics based on the frequency of no tumor growth in recipient animals 21 weeks after transplant, TIC frequency was determined to be reduced 3.6- and 2.7-fold (relative to the control ADC) in BR13 and BR22 tumors with corresponding P values of 0.0007 and 0.0186, respectively (Fig. 4B and C). These results demonstrate that PF-06647263 effectively reduces the TIC subpopulation in TNBC tumors, contributing to the observed sustained regressions.

TICs in subtypes of ovarian cancer express EFNA4 and can be killed by PF-06647263

Ovarian cancer shares several features with TNBC, including the common gain of chromosome 1q, where EFNA4 resides (37, 39, 40). To first determine whether TICs in ovarian cancer PDX tumor models were similarly enriched among CD324-expressing cells, tumors were phenotypically profiled, and subpopulations with or without CD324 expression were isolated and transplanted (Fig. 5A and B). In experiments across 10 distinct ovarian cancer PDXs of either the high-grade serous (HGS) or malignant mixed mullarian tumor (MMMT) subtype, 142 of 160 mice (89%) transplanted with 200 human epithelial CD324+ cells bore tumors within 24 weeks, versus only 9 of 172 mice (5%) transplanted with 200 CD324− cells (Supplementary Table S3).

As was observed for TNBC, the ovarian tumors that arose from CD324+ cells were histologically and phenotypically diverse. Like TNBC, CD324+ TIC from MMMT and a subset of HGS ovarian tumors expressed elevated levels of EFNA4 (Fig. 5C), which was confirmed by microarray gene expression profiling of bulk ovarian PDX tumors representing several subtypes (Fig. 5D; n = 31 HGS, 6 MMMT, and 13 "other"). As observed for TNBC, a substantial fraction (22.2%) of ovarian TCGA samples had significant EFNA4 copy-number gains (≥2.5; Supplementary Fig. S1B). As in TNBC, a correlation between EFNA4 mRNA level and DNA copy number was observed (r = 0.11; P < 0.0001), also...
suggested a potential genetic basis for overexpression in ovarian tumors (Fig. 5E).

Finally, to determine whether elevated EFNA4 expression in ovarian tumors could be leveraged therapeutically, naïve mice were implanted with PDX tumor cells, randomized, and treated with the standard-of-care therapeutic agents cisplatin or irinotecan (depending on OV PDX subtype), a control ADC, or PF-06647263. Significant PF-06647263–induced tumor regressions and/or TGI were observed in all ovarian PDXs evaluated (Table 1; n = 6; 3 HGS and 3 MMMT subtype). In 3 of 6 models tested, mice treated with a dose of 0.27 mg/kg were effectively cured, with no observed recurrences (Fig. 5F). The more sensitive models displayed significant TGI at doses as low as 0.036 mg/kg (Table 1). In contrast, the control ADC did not elicit antitumor activity in any study. Cumulatively, these data establish the robust single-agent activity of PF-06647263 in both TNBC and ovarian cancer PDX tumor models.

**Exploratory toxicology**

The nonclinical safety profile of PF-06647263 has been well characterized in repeat-dose studies (once every 3 weeks for 3 cycles) in cynomolgus monkeys. Overall, target organ toxicities observed in monkeys were attributed to target-independent (off-target) effects associated with the payload since they had been observed previously with other calicheamicin-based ADCs. Together, the exploratory toxicology results and in vivo efficacy data suggested that PF-06647263 would have a therapeutic window in cancer patients.

**Discussion**

For ADCs to reach their full potential, they must target appropriate cells and antigens, and leverage cytotoxic mechanisms equal to the task of eradicating target cells. TICs represent cellular targets that, if effectively eliminated, may result in a profound impact on patient survival. Like their normal stem cell counterparts, TICs likely contain a subpopulation of quiescent cells inherently resistant to cell-cycle–dependent microtubule inhibitors. Calicheamicin is a clinically validated toxin able to kill cells independent of their cell-cycle status (32). In preclinical efficacy studies, anti–EFNA4-ADC (PF-06647263) dramatically outperformed doxorubicin and cisplatin in TNBC and ovarian cancer PDXs, respectively, and together with the results of nonclinical toxicology and pharmacokinetic studies, provided rationale for the initiation of a phase I clinical trial. Previous clinical experience with an ADC that targets the EphA2 receptor (MEDI-547), with which EFNA4 ligand interacts, resulted in early and serious adverse events in humans (41). Both MEDI-547 and the normal tissue expression pattern of EphA2 are entirely different from PF-06647263 and EFNA4, and thus we do not expect, nor does evidence from preclinical studies suggest, that PF-06647263 will result in any of the same significant toxicities as MEDI-547.

Unlike small-molecule approaches to inhibit Ephrin/Eph receptor signaling, the ADC platform presents an opportunity to target specific Ephrin ligands. The Ephrin-A/EFNA subfamily of ligands is displayed on the cell surface via a glycosyl phosphatidylinositol (GPI) anchor (18). The ability of GPI-anchored proteins to internalize has been debated, yet several clinical-stage ADCs target such proteins (42–44). Consistent with requirement for ADCs to internalize to mediate payload delivery, we herein demonstrate that GPI-anchored EFNA4 efficiently internalizes to mediate payload delivery both in vitro and in vivo. Depending on the signaling functions of a particular Ephrin in tumors and normal tissues, the optimal mAb for an ADC against a particular Ephrin ligand may or may not functionally block Ephrin/Eph ligand.

**Figure 4.**

PF-06647263 eliminates TICs in vivo and causes sustained tumor regressions. A, Tumor growth curves for mice bearing the denoted PDX tumors following randomization and treatment Q7Dx2 with either vehicle (○) or 15 mg/kg doxorubicin (■), or BiVerx with 0.27 mg/kg (△) or 0.09 mg/kg (▲) PF-06647263 or 0.3 mg/kg control ADC (▲). Data, mean ± SEM of 6 to 8 mice per group. B, semilog plot graphing the percentage of non–tumor-bearing mice following serial transplantation of limiting dilutions of BR5 PDX human tumor cells 12 days after exposure to 0.09 mg/kg (▲) PF-06647263 or 0.3 mg/kg control ADC (▲). C, TIC frequency within the denoted PDX tumors following treatment with 0.1 mg/kg control or 0.09 mg/kg PF-06647263 is shown, wherein data represent the mean ± 1 standard error.
receptor interactions. In the course of characterizing anti-EFNA4 antibody clones, one that does not block receptor/ligand interactions exhibited greater efficacy than one that does. Considering the affinity of EFNA4 for all known EphA and EphB receptors is at least 10-fold lower than that of antibody for target (Supplementary Fig. S4), it is unclear why continued ligand engagement might be favored for PF-06647263 internalization.

Recent evidence suggests that EFNA4 may play a functional role in the cell fate decisions of mammary epithelial cells (45), signifying a biologic basis for the overexpression of EFNA4 in breast TIC. Moreover, the overexpression of EFNA4 in TNBC and ovarian cancer may have a genetic basis, as evidenced by the DNA copy-number gains observed in a subset of patient samples from both the queried breast and ovarian cancer TCGA and other similar datasets in the public domain. In light of observations that approximately 70% of non-Claudin low TNBC tumors are basal-like, and basal-like TNBC is more similar to HGS ovarian cancer than to other breast cancer subtypes (35, 39), it is perhaps not surprising that EFNA4 expression is also elevated in a subset of ovarian tumors. Strikingly, in hepatocellular carcinoma, copy-number gain of EFNA4 is even more frequent than in breast and ovarian cancer and is associated with higher mRNA levels (Supplementary Fig. S1C and S1D).

The ability to enrich for TNBC TICs using CD324 antigen expression was critical to revealing EFNA4 as a novel therapeutic target. Even when considering probable false-positive tumorigenicity events from CD324⁺/C0 cells in isolation and transplantation experiments, TNBC and ovarian TICs were functionally demonstrated to be enriched greater than 14- and 40-fold, respectively, upon isolation of CD324⁺ cells. Our in vivo demonstration that TNBC TICs are enriched by CD324 (E-cadherin, an epithelial marker) contrasts with in vitro data that TICs exhibit features of mesenchymal, but not epithelial, cells (46, 47). It seems likely that any association between TIC and the mesenchymal state could be

Table 1. Summary of preclinical efficacy studies with EFNA4-ADC

<table>
<thead>
<tr>
<th>Tumor subtype</th>
<th>PDX</th>
<th>Standard of care Regression (TTP, days) or % TGI</th>
<th>EFNA4 protein (ng/mg)</th>
<th>Dose level (mg/kg)</th>
<th>Regression (TTP, days) or % TGI</th>
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<tbody>
<tr>
<td>TNBC: Basal</td>
<td>BR5</td>
<td>44% TGI</td>
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<td>0.036</td>
<td>Regression (52)</td>
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<td></td>
<td>0.09</td>
<td>Regression (53)</td>
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<td>0.27</td>
<td>Regression (215)</td>
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<td></td>
<td>BR22</td>
<td>27% TGI</td>
<td>1.91</td>
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<td>No activity</td>
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<td></td>
<td>0.036</td>
<td>2% TGI</td>
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<td>55% TGI</td>
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<td></td>
<td>0.27</td>
<td>Regression (43)</td>
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<td></td>
<td>BR31</td>
<td>30% TGI</td>
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<td>Regression (91)</td>
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<td>0.036</td>
<td>34% TGI</td>
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<td>0.09</td>
<td>56% TGI</td>
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<td>0.27</td>
<td>Regression (60)</td>
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<td>TNBC: Normal-like</td>
<td>BR13</td>
<td>16% TGI</td>
<td>1.00</td>
<td>0.018</td>
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<td>0.036</td>
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<td>BR25</td>
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<td>No activity</td>
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<td>ND</td>
<td>0.27</td>
<td>No activity</td>
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<td>OV: HGS</td>
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<td>0.09</td>
<td>45% TGI</td>
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<td>Regression (49)</td>
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<td>Regression (53)</td>
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<td></td>
<td>OV63</td>
<td>35% TGI</td>
<td>0.49</td>
<td>0.036</td>
<td>No activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.09</td>
<td>59% TGI</td>
</tr>
<tr>
<td>OV: MMMT</td>
<td>OV45</td>
<td>Regression (20)</td>
<td>3.64</td>
<td>0.018</td>
<td>No activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.036</td>
<td>70% TGI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.09</td>
<td>Regression (60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.27</td>
<td>Regression (200)</td>
</tr>
<tr>
<td></td>
<td>OV55</td>
<td>NA</td>
<td>0.42</td>
<td>0.036</td>
<td>No activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.09</td>
<td>67% TGI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.27</td>
<td>Regression (42)</td>
</tr>
<tr>
<td></td>
<td>OV124</td>
<td>NA</td>
<td>ND</td>
<td>0.27</td>
<td>Regression (76)</td>
</tr>
</tbody>
</table>

Abbreviations: NL, normal-like; ND, no data.

*Breast SOC = 1.5 mg/kg doxorubicin q7dx2; Ovarian-HGS SOC = 5 mg/kg cisplatin q7dx2; Ovarian MMMT SOC = 35 mg/kg irinotecan q4dx3.
dependent on tumor subtype, microenvironmental context, and other parameters. Our results indicate that there is no such association in TNBC and underscore the importance of characterizing TIC by functional association in TNBC and other cancer subtypes. Our results indicate that there is no such association in TNBC and underscore the importance of characterizing TIC by functional association in TNBC and other cancer subtypes.

The use of CD324 to obtain a 14-fold enrichment for non-Claudin low TNBC TIC facilitated an 8.9-fold enrichment in non-Claudin low PDX tumors and supports the hypothesis that by effectively targeting and eradicating TIC, significant improvement to survival endpoints may be possible in patients.

The establishment and use of a large panel of well-annotated, low-passage PDX tumors enabled not only the enrichment and characterization of TIC using a novel marker, CD324, in breast and ovarian cancer subtypes, but also facilitated the discovery of EFNA4 as a therapeutic target. Moreover, the preclinical evaluation of anti–EFNA4-ADC (PF-06647263) in a simulated "mouse clinical trial" utilizing a variety of relevant PDX tumor models resulted in information guiding the clinical development strategy for this compound. PF-06647263 herein demonstrated robust antitumor activity in preclinical studies and is currently being evaluated in a phase I clinical trial.

Disclosure of Potential Conflicts of Interest
M. Damelin holds ownership interest (including patents) in Pfizer. A. Bankovich, M.A. Pyzj, J. Hampf, R. Stull, and S.J. Dylla hold ownership interest (including patents) in Stemcentix, Inc. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: M. Damelin, A. Bankovich, J. Aguilar, J. Lucas, P. Sapra, S.J. Dylla
Other (designed and made the calicheamicin conjugate of the anti EFNA4 that was used throughout this study): K. Khandke

Acknowledgments
The authors acknowledge contributions by the PF-06647263 Project Team, including but not limited to Ashwin Gollerkeri, Frank Loganzo, George Kan, Nahor Haddish-Berhane, Russell Dushin, Judy Lucas, Edward Rosfjord, Erik Upeslacis, Danielle Leahy, Marc Roy, Tania Franks, Leslie Ober, Steven Priet-Shepherd, Jonathon Golas, Andrea Hooper, Magali Guffroy, and Martin Finkelstein. They also thank Brett Pickell, Ayako Kuroda, Michelle Richardson, Peter Lindley, Michael Torgov, Gang Wang, Bob Liu, Somdutta Roy, and Saiyou Ohshima for their contributions.

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Received March 22, 2015; revised May 18, 2015; accepted May 19, 2015; published OnlineFirst May 26, 2015.
References


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